Inability of vascular smooth muscle cells to proceed beyond S phase of cell cycle, and increased apoptosis in symptomatic carotid artery disease

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Objective: **Microemboli passing through the cerebral artery downstream from high-grade carotid artery stenosis produce transient ischemic symptoms and may result in stroke. Rupture of carotid artery plaque is the main source of microemboli in high-grade internal carotid artery stenosis. However, the mechanisms underlying plaque rupture are unclear. We hypothesized that vascular smooth muscle cells (VSMC) from plaque in patients with symptoms of carotid artery stenosis undergo increased apoptosis and decreased proliferation, compared with VSMC in patients without symptoms.**

Methods: **VSMC were isolated by means of enzymatic dissociation from plaque removed at carotid endarterectomy in patients with symptoms of carotid artery stenosis, eg, hemispheric transient ischemic attacks, amaurosis fugax, or stroke, and patients with high-grade stenosis without symptoms. VSMC were cultured and immunostained with smooth muscle α-actin and caldesmon antibodies to ensure purity. TUNEL assay and annexin V labeling were performed to identify VSMC undergoing apoptosis. Proliferation assay with [^{3}H] thymidine incorporation was performed in VSMC stimulated with fetal bovine serum (FBS), and cell cycle profile was analyzed with DNA staining with Vindelov reagent.**

Results: **We isolated VSMC from symptomatic plaque that showed gross ulceration, and asymptomatic plaque. Apoptosis, as measured with the TUNEL assay, in VSMC from symptomatic plaque was 5.45% ± 0.8%, and in asymptomatic plaque was 1.20% ± 0.2%. Annexin V labeling revealed that 26.8% ± 3.8% cells were labeled for phosphatidylserine in VSMC in symptomatic plaque, compared with 4.8% ± 0.3% cells in asymptomatic plaque. VSMC in asymptomatic plaque showed significantly increased uptake of [^{3}H] thymidine at all concentrations of FBS, compared with symptomatic plaque. In the presence of 10% FBS, VSMC from asymptomatic plaque progressed through the S phase of the cell cycle, whereas significantly increased numbers of VSMC from symptomatic plaque were arrested in the S phase.**

Conclusion: **Increased numbers of VSMC from symptomatic plaque undergo apoptosis, compared with VSMC from asymptomatic plaque. This could be due to inability of VSMC from symptomatic plaque to progress beyond the S phase of the cell cycle. Decreased proliferation and increased loss of VSMC as a result of apoptosis in symptomatic plaque may result in plaque rupture, leading to development of symptoms. (J Vasc Surg 2003;38:155-61.)**

Microemboli generated from ruptured plaque are the main cause of cerebral symptoms in carotid artery disease.[1] To ensure symptom-free survival, the challenge is not simply to suppress progression of atherogenesis but to suppress symptoms caused by plaque rupture and ulceration in existing plaque. Three major determinants of plaque rupture are necrotic core size and consistency of lipid burden, fibrous cap thickness, and fibrous cap inflammation.[2] A lipid core occupying more than 40% of the plaque area[3] and a collagen-poor thin fibrous cap[4] are predisposing factors to plaque rupture. Ruptured fibrous caps have half as many vascular smooth muscle cells (VSMC) and twice as many macrophages as unruptured fibrous caps, indicating that the ratio of VSMC to macrophages is greatly altered, and the proportion of VSMC undergoing apoptosis is significantly increased in plaque in patients with unstable versus stable angina.[4,5]

VSMC are important in progression and stability of atherosclerotic plaque. VSMC produce extracellular matrix,[6] which contributes to strength of the fibrous cap and also is a vital component of the fibrous cap.[7] Macrophages and T-lymphocyte cytokines induce apoptosis in VSMC.[8] Loss of VSMC may reduce plaque cellularity and limit intimal thickening, which can also lead to rupture of the fibrous cap and thrombosis.[9] Rupture sites have low concentration of VSMC and increased concentration of macrophages and inflammatory cells.[10] Apoptosis of VSMC has been detected in the shoulder regions of plaques, sites that are predisposed to rupture.[11] VSMC have the same potency to generate thrombin as do platelets,[9] by expressing phosphatidylserine (PS) on the cell surface and in the presence of factor V and factor VII. PS acts as a substrate for thrombin production.[12] At present we have no evidence to support our conjecture linking apoptosis of VSMC and plaque

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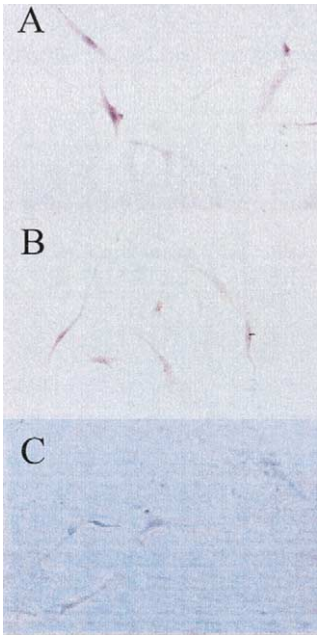

Fig 1. Specificity of vascular smooth muscle cells from carotid plaques for smooth muscle α-actin and caldesmon. Cells were isolated from atherosclerotic plaques of carotid artery and cultured. After the third passage, cells were stained with hematoxylin-eosin **(A)**, smooth muscle α-actin **(B)**, and caldesmon **(C)**. All the cells showed immunopositivity to α-actin and caldesmon, indicating that isolated cells were vascular smooth muscle cells, not macrophages or myofibroblasts.

rupture, because of limitations in animal models of plaque rupture and technical difficulties in identifying apoptotic VSMC in complex atherosclerotic plaque.

Previous studies have reported increased apoptosis and decreased survival of VSMC from advanced atherosclerotic plaque compared with VSMC cultured from normal artery.[13] However, VSMC from plaque in patients with and without symptoms were not differentially examined. In this study we tested our hypothesis that VSMC from symptomatic plaque had increased apoptotic and decreased proliferative profiles compared with VSMC from asymptomatic plaque, causing consequent loss of VSMC in the fibrous cap and leading to rupture.

MATERIAL AND METHODS

Patients and tissue collection

Tissue specimens were obtained from 4 patients with symptoms and 4 patients without symptoms, as identified from history and clinical examination performed by a vascular surgeon. These patients had 70% to 98% carotid artery stenosis and underwent carotid endarterectomy. Symptoms included hemispheric transient ischemic attacks, amaurosis fugax, or stroke. Time of collection of specimens from patients with symptoms was within 2 weeks of onset of symptoms. These plaques revealed ruptured fibrous caps and ulcerated luminal surface. No intraplaque hemorrhage was observed in any atherosclerotic plaque obtained from patients either with or without symptoms. The necrotic core of symptomatic plaque was considerably larger than that in asymptomatic plaque. At histologic evaluation with Gomori trichrome stain (data not shown), symptomatic plaque revealed decreased cellularity, with equivalent staining for collagen, compared with asymptomatic plaque. We reported increased immunoreactivity to CD68 in symptomatic plaque compared with asymptomatic plaque.[19] This observation suggested increased inflammation, primarily due to macrophage accumulation, in symptomatic plaque.

The Institutional Review Board of Creighton University approved the research protocol, and informed consent was obtained from patients.

Smooth muscle cell isolation and culture

VSMC were isolated from plaques with elastase and collagenase (Sigma, St Louis, Mo) digestion. VSMC from symptomatic plaque proliferated slower than those from asymptomatic plaque; the VSMC from asymptomatic plaque had a doubling time of approximately 40 hours, compared with approximately 60 hours for VSMC from symptomatic plaque. Confluent cells showed the characteristic hill-and-valley pattern associated with VSMC, and did not form swirls, which are characteristic of fibroblasts. Isolated cells were cultured serially in M199 media (Sigma), and subcultured strains were used between passages 3 and 7. We decided to use the cells up to passage 7 on the basis of changes in morphologic characteristics; after passage 7, VSMC from symptomatic plaque started to develop a rounded, hypertrophied appearance rather than their characteristic spindle shape. Purity of isolated VSMC was tested with positive immunostaining to smooth muscle α-actin (MO815; Dako, Carpenteria, Calif) and caldesmon (AM332-5M; Biogenex, San Ramon, Calif) (Fig 1). The remaining VSMC in passage 3 were frozen and maintained in liquid nitrogen for future use.

In-vitro apoptotic detection

TUNEL assay. VSMC were cultured in 25 cm^2 flasks and maintained for 24 hours in M199 medium and 0.5% to 1% fetal bovine serum (FBS). VSMC were then treated for 24 hours with either 2% or 10% FBS containing M199 medium. After 24 hours the cells were harvested with trypsinization, with 0.25% trypsin and 1 mmol/L of ethylenediaminetetraacetic acid (EDTA; Gibco/BRL, Grand Island, NY). Cells were fixed in 1% paraformaldehyde for 15 minutes on ice, and washed twice with phosphate-buffered saline solution (PBS). The cells were then permeabilized with ice-cold 70% ethanol for a minimum of 18 to 24 hours. All TUNEL assays were performed with an apolipoprotein–bromodeoxyuridine kit (Apo-BRDU kit; Pharmingen, San Diego, Calif). After washing the cells with wash buffer, cells were resuspended in 50 μL of labeling solution in flow cytometry tubes. Labeling of cells was terminated with centrifugation and washing of cells with rinse buffer; then 100 μL of anti BRDU–fluorescein iso-

thiocyanate (FITC) antibody solution was added to the cells. After 30 minutes of incubation in the dark, propidium iodide solution was added to cells. Flow cytometry was performed within 3 hours. At least 10,000 cells in each experimental group were analyzed in FACSCalibur (Becton-Dickinson, San Jose, Calif) with Lysis II software.

Annexin V labeling. For detection of PS externalization, VSMC in culture were collected after the passage 3. Adherent cells were washed twice with PBS (pH 7.4) trypsinized with 0.25% trypsin and 1 mmol/L of EDTA. The isolated cells in the medium were centrifuged at 1500 rpm for 10 minutes at 4° C. The pellet was resuspended in 5 mL of cold PBS and centrifuged. An annexin V–FITC kit (Pharmingen) was used to measure PS externalization. The incubation buffer (10 mmol/L of HEPES [*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid]–potassium hydroxide [pH 7.5], 140 mmol/L of sodium chloride, 2.5 mmol/L of calcium chloride) was added to the cells, which were then incubated in the presence of 10 μg/mL annexin V–FITC and 5 μg/mL of propium iodide for 15 minutes at 37° C. Labeled cells were analyzed with flow cytometry with FACSCalibur and Lysis II software. In each experimental group 10,000 cells were counted, and these events were thought to represent at least 90% to 95% of the total cell population.

Proliferation assay

VSMC were maintained in M199 medium containing 10%FBS. Cells were harvested, and 10,000 cells per well were added to a 12-well plate. VSMC were maintained in culture media supplemented with 10% FBS for 24 hours, followed by culture in serum-deprived medium (M199 medium containing 0.5% to 1% FBS) for 24 hours. After quiescent cells in the treatment groups received M199 medium conditioned with 2%, 10%, or 20% FBS, [^{3}H]thymidine was added in the last 6 hours of the 24 hours of conditioning. Cells were washed three times with PBS and digested with 1 mL of 1N NaOH. Radioactivity in each well was counted in a β-scintillation counter and standardized between groups with protein concentration.

Cell cycle profile

Cells were grown to 90% to 95% confluency and trypsinized. The cells were then pelleted, resuspended, and washed. Washed cells were passed through a cell sorter, and the number of cells was determined. To every 1×10^5 cells, 1 mL of Vindelov reagent (containing Tris-buffered saline solution, ribonuclease A [type IIA, 70 U/mg; Sigma], PI [Sigma], nonidet P-40 [Sigma]) was added, and the cells were placed in a tube covered with foil to protect against light and placed in a refrigerator at 4° C. The cells were analyzed in a flow cytometer to determine cell cycle phase of each cell. Lysis II software was used to collect the data. In each experiment, 10,000 cells were counted, and these events are thought to represent at least 90% to 95% of the total cell population. The area versus the width of the fluorescent signal was analyzed to gate out cellular multiples. Histograms of DNA content were analyzed with Modfit Lt V1.01 (Verity Software House, Topsham, Me) to determine fractions of the population in each phase of the cell cycle (G_0, G_1, S, and G_2/M).

Table I. Apoptotic index in vascular smooth muscle cells from symptomatic and asymptomatic plaque

	*Apoptotic index**	
	TUNEL	*Annexin V*
Asymptomatic	1.20 ± 0.2	4.8 ± 0.3
Symptomatic	5.45 ± 0.8†	26.8 ± 3.8†

*Calculated as ratio of immunostained cells (with dUTP in case of TUNEL or annexin V antibody) to total number of cells times 100.
Ten thousand events were counted with flow cytometry. Values represent mean ± SEM (n = 4) in each experimental group.
†$P < .05$.

Statistical analysis

Statistical analysis was performed with Prism 3.0 (Graphpad, San Diego, Calif). Data were calculated as mean plus or minus standard error of the mean, and were analyzed with the unpaired Student *t* test. Multiple comparisons were analyzed with analysis of variance to compare individual experimental groups. $P < .05$ was considered significant.

RESULTS

Apoptotic death in VSMC from asymptomatic and symptomatic plaque. Cultured VSMCs from symptomatic plaque showed spontaneous apoptosis even in high serum–containing medium, whereas VSMC from asymptomatic plaque continued proliferation and survived in low-serum media. Untreated VSMC consistently showed minimal autofluoresence (1.3%-2.0%). Furthermore, in the presence of annexin V–FITC or PI alone, there was a similar pattern of autofluorescence (1.2%-2.6%). With M199 medium with 10% FBS, the number of annexin V–positive cells was significantly greater ($P < .01$) in VSMC isolated from symptomatic plaque (26.8% ± 3.8%) compared with asymptomatic plaque (4.8% ± 0.3%) (Table I). It was difficult to measure apoptosis in VSMC from symptomatic plaque maintained in M199 supplemented with 2% FBS, because of excessive fragility of the cells and high cell debris.

Annexin V labeling indicates initial response to apoptotic stimuli. To determine DNA fragmentation that occurs during apoptosis, we performed the TUNEL assay to identify 3′-OH nick ends of the DNA with deoxyuridine triphosphate (dUTP) labeling. VSMC were grown under similar culture conditions as described for annexin V labeling. VSMC from asymptomatic plaque had a significantly higher ($P < .05$) degree of dUTP labeling (5.45% ± 0.8%) compared with VSMC from symptomatic plaque (1.20% ± 0.2%) (Table I).

Proliferation in VSMC from asymptomatic and symptomatic plaque. Thymidine is an important component of cell DNA, and when a cell proliferates it makes

Table II. Cell proliferation and cell cycle profile in vascular smooth muscle cells from symptomatic and asymptomatic plaque

	Cell proliferation ($\times 10^4$ cpm)*			*Cell cycle profile† (% VSMC)*	
	2% FBS	*10% FBS*	*20% FBS*	*S phase 1% FBS*	*S phase 10% FBS*
Asymptomatic	10.6 ± 0.5	16.4 ± 0.3	24.4 ± 1.4	39.1 ± 3.2	10.5 ± 4.2
Symptomatic	8.9 ± 0.1‡	13.9 ± 0.3‡	16.5 ± 0.3*	41.3 ± 5.1	40.8 ± 10.1*

*Values represent mean ± SEM (n = 4).
†Values shown as percent number of cells in S phase as mean ± SEM (n = 4).
‡$P < .05$

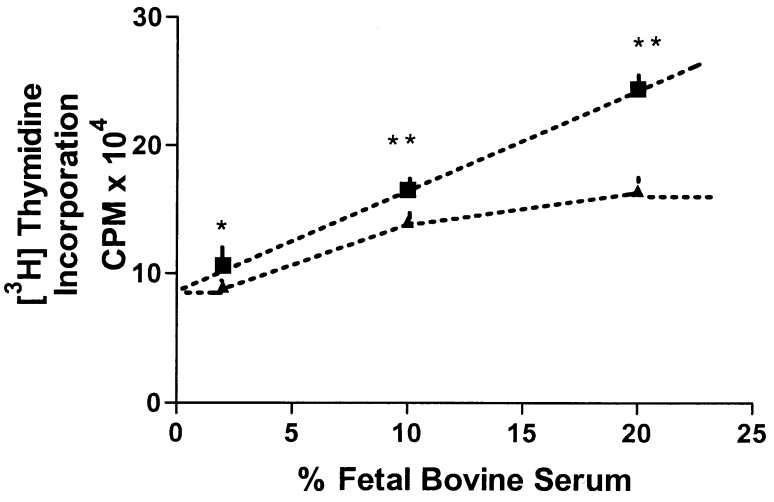

Fig 2. Thymidine incorporation in VSMCs from asymptomatic and symptomatic plaques after stimulation with 2%, 10%, and 20% FBS for 24 hours in a 37°C humidified 5% CO_2 incubator. Data are shown as the mean ± SEM from four symptomatic and asymptomatic plaques. $**P < .002$ and $*P < .05$ (*square* represents asymptomatic VSMCs and *triangle* represents symptomatic VSMCs).

DNA to supplement two cells. Therefore, by measuring incorporation of radioactive-labeled thymidine we can determine whether the cells are actively proliferating or are quiescent. These experiments were run in the isolated VSMC of symptomatic and asymptomatic plaques. We examined whether increasing the external stimuli for survival by supplementing the medium with higher FBS concentration would help the cells overcome apoptotic stimuli and show greater proliferation.

We examined proliferation of VSMC under three concentrations of FBS: 2%, 10%, and 20% (Table II). In all three concentrations of FBS there was significantly lower thymidine incorporation in the VSMC of symptomatic plaque compared with asymptomatic plaque. However, the most striking difference was observed in 20% FBS– supplemented media (Fig 2) ($P < .002$). Further analysis of the data revealed that VSMC of asymptomatic plaque continued proliferating with increasing concentration of FBS, whereas proliferation of VSMC of symptomatic plaque appeared to reach a plateau at 10% FBS (Fig 2).

Cell cycle profile of VSMC from symptomatic and asymptomatic plaque. Our experiments in cell phase analysis showed that VSMC from symptomatic plaque were incapable of progressing through the cell cycle and could not proceed beyond the S phase. Fig 3 shows representative histograms of the DNA content of VSMC of symptomatic and asymptomatic plaques after treatment for 48 hours with M199 supplemented with 1% FBS. Under these serum-deprived conditions, there was no significant difference in the fraction of cells in the S phase from asymptomatic plaque (41.3% ± 5.1%; Fig 3, *A*) and symptomatic plaque (39.13% ± 3.20%; Fig 3, *B*). However, with serum stimulation with 10% FBS, VSMC of asymptomatic plaque proceeded through the S phase into the G_2 phase, thereby decreasing the number of cells in the S phase (10.48% ± 4.2%; Fig 3, *C*). Of interest, there was no effect of 10% FBS on number of VSMC from symptomatic plaque in the S phase (40.8% ± 10.1%; Fig 3, *D*). Numbers of cells in the S phase from symptomatic plaque were similar under conditions of both serum stimulation and serum deprivation.

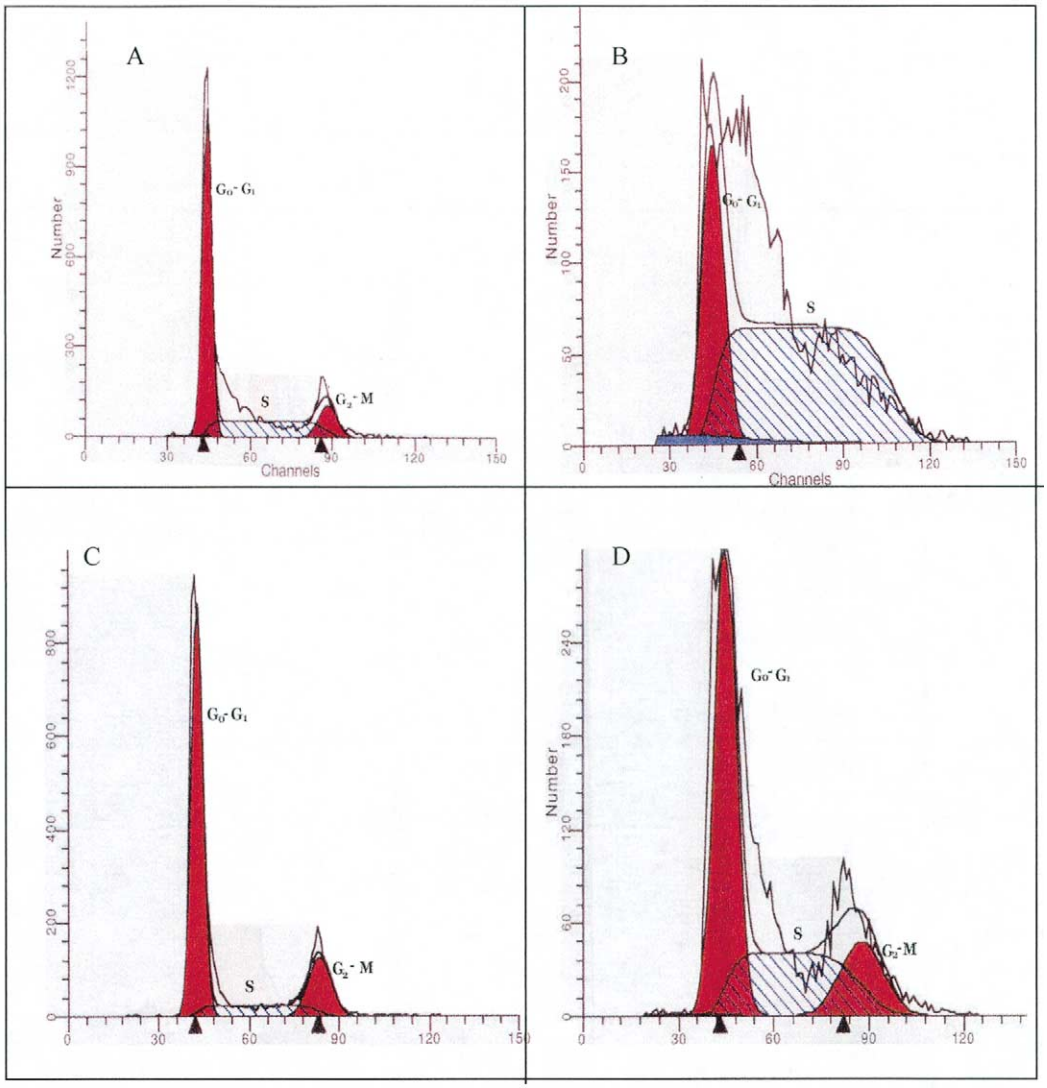

Fig 3. Cell proliferation profile of VSMCs from asymptomatic and symptomatic plaques. Representative histograms of DNA content as stained with Vindelov's reagent in VSMCs isolated from asymptomatic (**A** and **C**) and symptomatic (**B** and **D**) plaques. VSMCs were incubated with 1% FBS (**A** and **B**) or 10% FBS (**C** and **D**) for 48 hours before measuring the DNA content. The red peaks on each panel represent G_0/G_1 peak on the left and G_2/M phases on the right. The zone indicated by bars is the S phase. Based on their DNA content VSMCs collect in channels and the events in each phase are recorded. The Y axis indicates the total events in each phase while the X axis is indicative of the cell cycle phases. Please note a difference in the scale on the Y axis in **A** and **C** versus **B** and **D**.

DISCUSSION

Apoptosis is tightly linked with the cell regulatory pathway.[14,15] Any defect or error in the cell regulatory pathway results in apoptosis of the cell. Several studies have reported on apoptotic cell death in VSMC cultured from atherosclerotic coronary and aortic arteries.[11,16,17] Apoptosis in VSMC of carotid artery plaque has also been identified, but no studies have been reported that compare VSMC isolated from plaque of patients with symptomatic and asymptomatic carotid stenosis.

According to the North American Symptomatic Carotid Endarterectomy Trial (NASCET) and the published guidelines for carotid endarterectomy, patients with more than 70% carotid stenosis and highly echogenic plaque are ideal candidates for carotid endarterectomy.[18] Patients with or without symptoms might have similar stenosis (>70%) in the carotid artery. However, it is unclear why in one group of patients plaque ruptures and breaks off to produce symptoms. Therefore the availability of plaque from both asymptomatic and symptomatic patients offers

an excellent opportunity to investigate possible underlying mechanisms that may drive stable asymptomatic plaque to fissure or rupture. We reported a higher percentage of apoptotic nuclei and a thinner fibrous cap in symptomatic plaque compared with asymptomatic plaque.[19] This suggests that these factors might be involved in destabilizing plaque, causing rupture and leading to symptomatic carotid disease.

In this study we compared the apoptotic and cell proliferative pathways in VSMC from symptomatic and asymptomatic plaque. In our previous study we observed a higher number of apoptotic cells in symptomatic plaque compared with asymptomatic plaque. Because of limitation in identifying the cell type undergoing apoptosis, we decided to isolate and culture VSMC from plaque, because they have a pivotal role in maintaining plaque stability. We observed spontaneous death of VSMC from symptomatic plaque, whereas VSMC from asymptomatic plaque remained viable and continued proliferating. Furthermore, VSMC from symptomatic plaque were difficult to maintain in culture with minimum serum stimulation, indicating that growth factor stimulation is required to maintain viability of VSMC from symptomatic plaque. These data suggest that VSMC from symptomatic plaque may have the intrinsic property of undergoing apoptosis as a result of loss of telomerase enzyme.[20] Bennett et al[13] reported similar observations in VSMC cultured from advanced atherosclerotic lesions of coronary arteries.

Apoptosis of VSMC is complex and has positive and negative outcomes on progression of disease. Apoptosis of VSMC leads to decrease in VSMC population, thereby decreasing intimal thickening, as shown in animal models.[21] On the other hand, loss of VSMC leads to decrease in collagen production and thinning of the fibrous cap.[22] Therefore, for stable plaque it is vital to have a balance in programmed cell death and proliferation of VSMC.

To test whether VSMC from symptomatic plaque, although they show a higher apoptotic index, are also capable of undergoing proliferation, we performed a cell proliferation assay. We observed that VSMC from asymptomatic plaque had a healthier phenotype, with increased thymidine incorporation with increased serum stimulation. Although we observed a similar trend in VSMC from symptomatic plaque, general incorporation of thymidine by these cells was lower than that from asymptomatic plaque. Although thymidine incorporation indicates that a cell is capable of synthesizing DNA, it does not provide evidence for completion of the cell cycle and resultant mitosis. Therefore we performed analysis for cell cycle profile.

Most VSMC from symptomatic plaque were arrested in the S phase of the cell cycle, which could explain why we observed an increase in thymidine incorporation without efficient cell turnover. Thus the cell made enough DNA, as measured by thymidine incorporation, but was incapable of dividing into two cells. This suggests that VSMC from symptomatic plaque had a dysfunction in the cell cycle regulatory pathway, whereby the cells were capable of being stimulated to enter the cell cycle but lacked the internal machinery to carry the cell beyond the S phase. This can be due to deficiency in the intermediate cell cycle proteins, eg, cyclins and enzymes, required to proceed through G_2 and M phases.[23] Alternatively, since the cells could have already reached senescence, further serum or growth factor stimulation drives the cells to undergo apoptosis.[24]

Atherosclerotic plaque and its components are complex, and it is difficult to identify a single growth factor or mitogen that can trigger apoptosis on senescent cells. We previously reported that insulin-like growth factor–1 (IGF-1) has a major role in VSMC survival.[25] It is therefore possible that VSMC from symptomatic plaque have decreased density or affinity of IGF-1 receptors. Alternatively, there could be a decreased receptor-response coupling activation of IGF-1 receptors. In this regard, IGF-1 binding proteins and other factors within the transmembrane signaling pathway may have a substantial role. This could be supported by our preliminary studies, in which area of immunoreactivity to IGF-1Rα antibody in necrotic core, fibrous cap, and media was significantly greater in asymptomatic plaque compared with symptomatic plaque (unpublished data).

Findings from our studies provide valuable insight into the survival of VSMC. It is now imperative to unravel events that condition VSMC from symptomatic plaque to undergo apoptosis. There have been some reports that VSMC express FasL[24]. VSMC express Fas receptor,[24] and therefore it will be interesting to identify expression of Fas/FasL on VSMC from symptomatic and asymptomatic patient plaque. Second, the question as to which apoptotic pathway the cells choose for undergoing apoptosis will also be a key factor in understanding apoptosis in VSMC isolated from atherosclerotic plaque and in maintaining the balance between apoptosis and proliferative responses, which in turn may provide plaque stability.

In summary, data from this study suggest that increased apoptosis of VSMC from symptomatic plaque is most likely caused by arrest of the cells in the S phase, which could be due to excessive cell cycling of VSMC or to loss of telomerase enzyme, leading to senescence. Increased apoptosis of VSMC in symptomatic plaque could also be a result of plaque rupture rather than an event preceding plaque rupture. A better understanding of the pathways involved in apoptosis of VSMC in carotid artery stenosis will provide opportunities for development of more precise and cost-effective treatment.

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